Research Article

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Immunogold labelling to localize polyphenol oxidase (PPO) during wilting of red clover leaf tissue and the effect of removing cellular matrices on PPO protection of glycerol-based lipid in the rumen

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Abstract

BACKGROUND: The enzyme polyphenol oxidase (PPO) reduces the extent of proteolysis and lipolysis within red clover fed to ruminants. PPO catalyses the conversion of phenols to quinones, which can react with nucleophilic cellular constituents (e.g. proteins) forming protein–phenol complexes that may reduce protein solubility, bioavailability to rumen microbes and deactivate plant enzymes. In this study, we localized PPO in red clover leaf tissue by immunogold labelling and investigated whether red clover lipid was protected in the absence of PPO-induced protein–phenol complexes and plant enzymes (lipases).

RESULTS: PPO protein was detected to a greater extent ($P < 0.001$) within the chloroplasts of mesophyll cells in stressed (cut/crushed and wilted for 1 h) than freshly cut leaves for both palisade (61.6 and 25.6 Au label per chloroplast, respectively) and spongy mesophyll cells (94.5 and 40.6 Au label per chloroplast, respectively). Hydrolysis of lipid and C18 polyunsaturated fatty acid biohydrogenation during *in vitro* batch culture was lower ($P < 0.05$) for wild-type red clover than for red clover with PPO expression reduced to undetectable levels but only when cellular matrices containing protein–phenol complexes were present.

CONCLUSION: Damaging of the leaves resulted in over a doubling of PPO detected within mesophyll cells, potentially as a consequence of conversion of the enzyme from latent to active form. PPO reduction of microbial lipolysis was apparent in macerated red clover tissue but not in the absence of the proteinaceous cellular matrix, suggesting that the PPO mechanism for reducing lipolysis may be primarily through the entrapment of lipid within protein–phenol complexes.

Keywords: polyphenol oxidase; immunogold; mesophyll cells; lipolysis; biohydrogenation

INTRODUCTION

Polyphenol oxidases (PPO) of plants and fungi are copper metalloenzymes which carry out hydroxylation of monophenols to diphenols and the oxidation of diphenols to quinones using molecular oxygen. These PPO-generated quinones are highly reactive and can readily bind covalently with nucleophilic sites, e.g. on amino acids, resulting in the formation of cross-linked protein polymers.[1] PPO can exist in either an active or a latent state and during periods of stress the latter is converted to the former.[2] It has been demonstrated that one route to activation of latent red clover (*Trifolium pratense*) PPO appears to be by its endogenous phenolic substrates: phaselic acid and clovamide.[2] However, while constitutive red clover PPO is predominantly in the latent form, *in vivo* substrate activation is prevented in healthy tissue by the separate subcellular compartmentation of the enzyme, which resides in the chloroplast,[3] and its substrates phaselic acid and clovamide, which are presumed to reside in the vacuole. Using transmission electron microscopy (TEM) and immunogold labelling with antibody raised against sodium dodecyl sulfate (SDS)-solubilized red clover PPO1 gene product,[4] we examined any changes in PPO localization in plant leaf mesophyll cells during induced stress (crushing and wilting) to determine whether obvious mixing of enzyme and substrate occurred that might be required to activate PPO.

The interest in PPO in animal fodder crops such as red clover is related to their potential to increase nitrogen use efficiency[5,6] and the supply of beneficial polyunsaturated fatty acids (PUFA) in ruminant products.[6,7] PPO has been shown to reduce both proteolysis and lipolysis in incubated red clover.[2,4,8] One possible

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mechanism whereby this is accomplished could be through deactivation of plant hydrolytic enzymes (proteases and lipases) as a consequence of quinone binding, since it has been shown that exogenously added proteases are inactivated in the presence of red clover extract.[9] More recently, Lee *et al.*[10] showed that tissue from genetically modified red clover with the PPO1 gene silenced[4] and no detectable PPO activity had significantly higher levels of lipolysis than that from wild-type red clover with normal levels of PPO activity in the presence of rumen microorganisms under anaerobic conditions. As PPO requires molecular oxygen for its enzymatic activity, it seems unlikely that PPO could be exerting its effect on lipids by inactivating lipolytic enzymes from anaerobic ruminal microbes and suggests PPO is indirectly affording some level of protection to the red clover glycerol-based membrane (GBM) lipid from hydrolytic enzymes. We hypothesize that ruminal lipid protection is the result of protein–quinone complexes, formed by the action of PPO, surrounding GBM lipid micelles and preventing access by lipolytic enzymes present in the rumen. This study investigated whether red clover GBM lipids are protected in the absence of such protein complexes and thus provides support for such a mechanism by which PPO reduces ruminal lipolysis.

MATERIALS AND METHODS

Experimental design and aim

Immunogold labelling was used to localize PPO protein within chloroplasts of mesophyll cells in fresh and stressed (crushed and wilted) leaf tissue to determine whether cellular integrity prevented formation of active PPO. Extracted lipid (i.e. free from cellular protein) from red clover tissue from plants with either wild-type levels of PPO or PPO reduced to undetectable levels by RNA interference were incubated in the presence of rumen inoculum alongside red clover tissue from the same plants which contained lipid and other cellular matrices such as PPO-induced protein–phenol complexes. Lipolysis was then measured to determine whether removal of the cellular matrix affected the ability of PPO to protect GBM lipid.

Plants and treatments

Plant material used was derived from a cross between a clone having normal levels of foliar PPO activity derived from WI-2 red clover germplasm[4] and NRC27-64-1 (T_o), a red clover plant having undetectable levels of PPO activity due to transformation with a pHannibal-based gene silencing construct.[11] The resulting T_1 seeds were sown in individual pots containing John Innes No. 2 loam-based compost and inoculated with rhizobium culture 2 days later. The plants were grown and maintained under controlled conditions (temperature of 20/15 °C, photoperiod of 16 h, and humidity of 0.6 kPa at both temperatures). Presence or absence of the silencing transgene was determined by standard polymerase chain reaction techniques using the primers 5′-AGTTGGGAAATTGGGTTCGAAATCG-3′ and 5′-TCATTAAAGCAGGACTCTAGAGGATC-3′, which anneal to the pdk intron (sense) and OCS terminator (antisense) regions of the pHannibal vector to amplify the antisense arm of the construct. The PPO phenotype of individual plants was determined by the quantitative assay described below. Four T_1 plants lacking the transgene and having wild-type levels of PPO activity were designated as PPO+, whereas nine plants containing the transgene and having undetectable levels of PPO activity were designated as PPO−.

A description of the experimental treatments is given in Fig. 1. For this current study, the PPO+ and PPO− red clover plants were on a 6-week regrowth before harvesting at 5 cm above soil level to give ∼200 g fresh weight (FW) of each. A sample of the fresh PPO+ material (∼5 g) was taken for PPO localization using immunogold labelling before the rest of the material was crushed using a rolling pin and board, cut into 5 mm strips and left on the laboratory bench for 1 h. A subsample of PPO+ leaf material (∼5 g) was again taken for PPO localization determination as with the fresh material before both PPO− and PPO+ material were frozen with liquid N_2 and split into two equal portions (∼100 g FW). Half was stored at −20 °C and subsequently used as the protein complex treatment (PC). The second half of the material was freeze-dried, ground and 1 g dry matter (DM) weighed into 12 extraction tubes for each treatment (PPO− and PPO+) to give 24 tubes in total. The remaining freeze-dried material was used for chemical analysis as described under 'Chemical analysis and PPO assay' below. Lipid was extracted from the freeze-dried samples weighed into the 24 extraction tubes by adding 4 mL of chloroform–methanol (2 : 1; v/v) and vortexing. These were centrifuged at 2000 × *g* and the solvent layers transferred to clean incubation tubes. An additional 4 mL of chloroform–methanol (2 : 1; v/v) was added to the freeze-dried material in each tube, the tubes were vortexed and centrifuged as before, and the solvent from the extraction pooled with the previous extract. The extraction was repeated once more so that three solvent layers were pooled. These were dried under N_2 at 50 °C and, once dry, protein, water-soluble carbohydrate and fibre were added in the form of Zein (0.26 g), glucose (0.08 g) and cellulose (0.30 g) to produce the free lipid treatment (FL), which was stored at −20 °C until needed. The proportions of each nutrient added to the FL were determined from the mean analyses of the freeze-dried PPO+ and PPO− material. On the day of the experiment the PC material was defrosted and, based on the calculated freeze-dried matter of the red clover, sufficient material to account for 1 g DM extracted for the FL treatment (∼5 g FW) was weighed into 12 incubation tubes for both PPO− and PPO+ to yield a further 24 tubes.

On the day before the weighing out of the PC treatments, anaerobic incubation medium was made up as described by Goering and Van Soest[12] and placed in a water bath at 39 °C overnight with continual CO_2 purging. The next day hand-squeezed rumen fluid (1 L) was collected from four rumen-fistulated dairy cows maintained on permanent pasture, and the fluid transferred back to the laboratory in a temperature-regulated flask (39 °C). Anaerobic buffer (7.5 mL) was dispensed into each of the 48 incubation tubes (containing PC PPO+, PC PPO−, FL PPO+ or FL PPO−) by peristaltic pump while gassing the headspace with CO_2 as the tubes were sealed. Each tube was reduced with 0.35 mL of reducing agent (containing 0.6% cysteine HCl, 0.6% Na_2S, 4% 1 mol L^{-1} NaOH)[12] and inoculated with 2.5 mL of the strained rumen fluid.

Incubations and lipid analysis

The tubes were incubated in the dark at 39 °C and a set of three tubes harvested at 0, 2, 6 and 24 h for each treatment. At these time points the supernatant from the PC treatments were subsampled for ammonia-N (1 mL mixed with 100 µL of 2 mol L^{-1} HCl and maintained at −20 °C) and free amino acid (FAA) analyses (1 mL maintained at −20 °C). All incubation tubes (PC and FL) were then frozen with liquid N_2 and the contents freeze-dried. Once freeze-dried the lipid was extracted as previously described for the preparation of FL but with the addition of 100 µL of

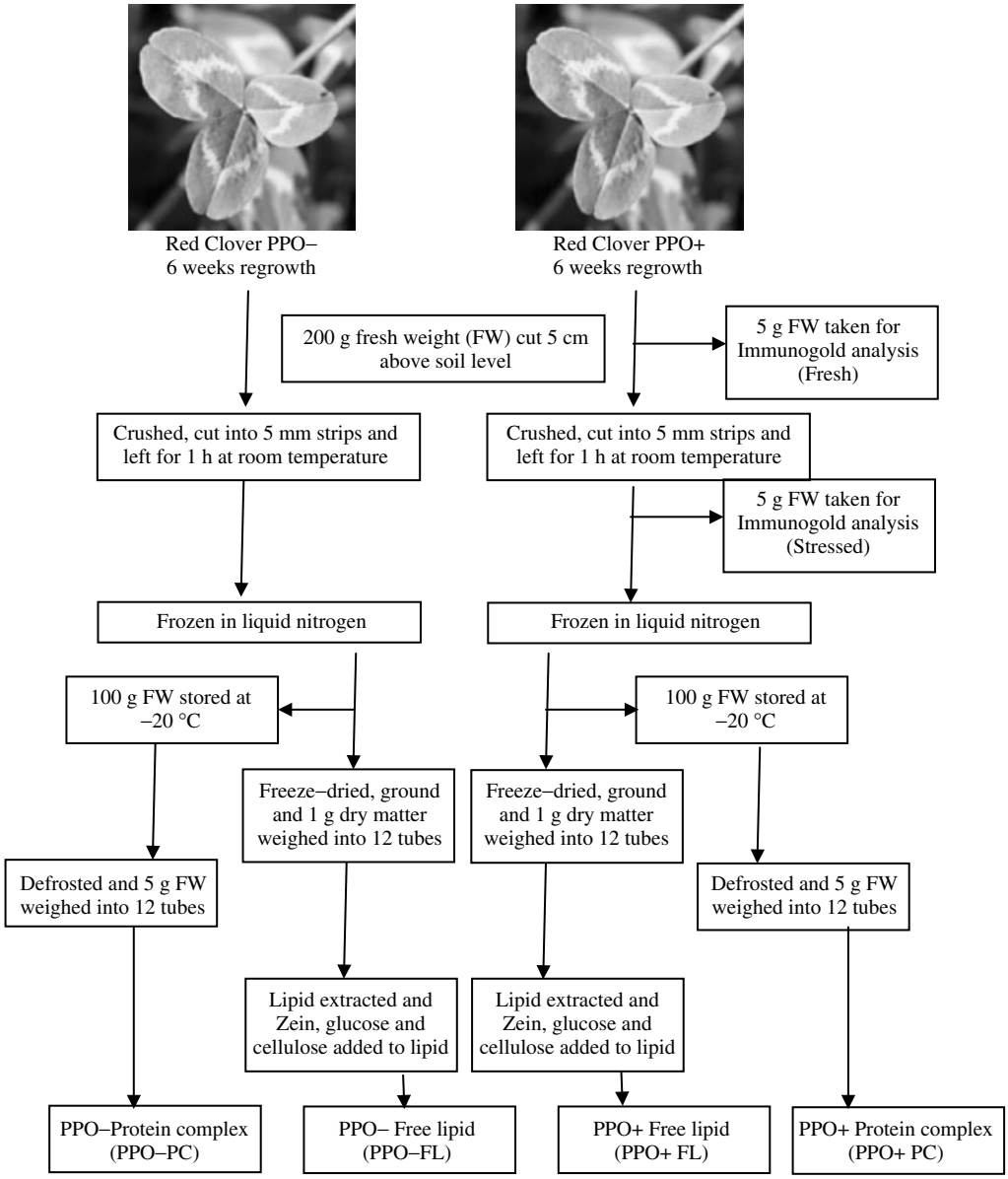

Figure 1. Treatment set-up procedure schematic.

internal standard (C21 : 0 15 mg mL^{-1} $CHCl_3$) to each tube. The chloroform–methanol extract was then split into two portions, with one portion transferred to a clean tube for lipid fractionation by thin-layer chromatography (TLC)[13] to separate the lipid into four fractions: GBM lipid (phospho- and galacto-lipid), triacylglycerol (TAG), diacylglycerol + monoacylglycerol (DAG + MAG) and free fatty acids (FFA). Each collected fraction was extracted from the TLC plates and converted into fatty acid methyl esters (FAME) by the addition of 4 mL of 0.5 mol L^{-1} HCl in methanol and 2 mL of toluene containing 0.4 mg mL^{-1} C23 : 0 as an internal standard and heating for 2 h at 50 °C. The second portion (i.e., not used for TLC) was dried under N_2 at 50 °C, resuspended in 1 mL of heptane, and converted to FAME using the bimethylation procedure (1.4 mol L^{-1} HCl in methanol and 0.5 mol L^{-1} NaOH in methanol) of Kramer and Zhou[14] to give the total fatty acid content of each sample, which was used to calculate biohydrogenation of the C18 PUFA. FAME were analysed by gas–liquid chromatography on a CP Sil 88 FAME column (100 m × 0.25 mm i.d., Chrompack UK Ltd, London, UK) with split injection (30 : 1). Peaks were identified from standards (ME61, Larodan Fine Chemicals, Malmo, Sweden; S37, Supelco, Poole, UK) and quantified using the internal standard (C21 : 0 for total fatty acid analyses and C23 : 0 for lipid fractionation analyses).

Ammonia-N and free amino acid analysis

Ammonia-N was assessed colorimetrically using indophenol blue,[15] on a Segmented Flow Analyser (ChemLab Instruments Ltd, Hornchurch, UK). Total FAA content was calculated using the ninhydrin method of Winters *et al.*[16]

PPO localization: immunogold labelling

PPO localization was carried out using transmission electron microscopy (TEM) and immunogold labelling of PPO. Leaf discs (~2 × 1 mm) of fresh and stressed (crushed and wilted for 1h) PPO+ tissue were cut from the centre of red clover leaflets,

avoiding the midrib and leaf margins. The discs were fixed in a primary fixative containing 3% glutaraldehyde (Agar Scientific, Stansted, UK) in 50 mmol L^{-1} PIPES (piperazine-*N*,*N*′-bis[2-ethane sulfonic acid], Sigma-Aldrich, Gillingham, UK) buffer overnight at 4 °C. The next day the fixative was removed with 50 mmol L^{-1} PIPES wash buffer (pH 7.4) and two changes of 0.1 mol L^{-1} sodium cacodylate (Agar Scientific) buffer (pH 7.2). The samples were then treated with a secondary fixative consisting of 1% osmium tetroxide (Agar Scientific) and 0.68% imidazole[17] (Sigma-Aldrich) in 0.1 mol L^{-1} sodium cacodylate buffer for 2 h. The fixative was removed with two changes of 0.1 mol L^{-1} sodium cacodylate wash buffer. The leaf discs were dehydrated in a standard ethanol series, followed by propylene oxide and embedded in TAAB Emix hard-grade resin overnight at 60 °C (both TAAB Laboratories Equipment Ltd, Aldermaston, UK). Ultrathin 60–80 nm sections were cut on a Reichert-Jung Ultracut E ultramicrotome with a Diatome Ultra 45 diamond knife and collected on Gilder GS2X0.5 3.05 mm diameter nickel slot grids (Gilder Grids, Grantham, UK) float-coated with Butvar B98 polymer[18] (Agar Scientific) films. The sections were immunolabelled by a two-step indirect method. Aldehyde quenching was achieved by room-temperature incubation in standard saline citrate + 0.015% Tween-20 (SSC-T) for 30–60 min. Non-specific binding was reduced by immersion at ambient temperature in casein–thiomersal buffer (CTM-T)[19] for 30–60 min. Red clover PPO rabbit (*Sylvilagus floridanus*) antiserum[4] was applied at a ratio of 1 : 200 in CTM-T overnight at 4 °C. After washing in carbon dioxide-free distilled reverse-osmosis water using microwave irradiation[20] and reblocking with CTM-T, sections were incubated at room temperature in EMGAR.15 (EM goat (*Capra aregagrus*) anti-rabbit IgG:15 nm gold; British Biocell, Cardiff, UK) at a ratio of 1 : 50 in CTM-T for 1–2 h and microwave-washed four times. Experimental controls included substitution of the PPO-specific antibody with pre-immune serum or CTM-T buffer and substitution of the EMGAR.15 immunogold conjugate with EMDAS.15 (donkey (*Equus africanus*) anti-sheep (*Ovis aries*) IgG:15 nm gold; British Biocell). All sections were double-stained with uranyl acetate[20] (Agar Scientific) and Reynold's lead citrate[21] (TAAB Laboratories Equipment Ltd, Aldermaston, UK) and observed using a JEOL JEM1010 transmission electron microscope (JEOL Ltd, Tokyo, Japan) at 80 kV. The resulting images were photographed using Kodak 4489 electron microscope film (Kodak Ltd, Hemel Hempstead, UK) developed in Kodak D-19 developer for 4 min at 20 °C, fixed, washed and dried according to the manufacturer's instructions.

The resultant images were analysed by selecting 50 intact chloroplasts at random in two cell types (spongy mesophyll and palisade mesophyll) and counting the Au label. For the controls, replacing the PPO1 antiserum primary antibody with plain CTM-T resulted in no immunogold labelling, as did replacing EMGAR.15 with EMDAS.15. Replacing the PPO1 antiserum with the pre-immune serum resulted in a low level of immunogold labelling, which was used as the blank with the mean pre-immune counts subtracted from the PPO1 antiserum counts.

Chemical analysis and PPO assay

Water-soluble carbohydrate (WSC) of the freeze-dried red clover was determined spectrophotometrically using anthrone in sulfuric acid on a Technicon Autoanalyser (Technicon Corporation, New York, USA).[22] Ash and by mass difference organic matter (OM) were analysed by combusting the ground samples at 550 °C for 6 h in a muffle furnace. Total nitrogen was determined by micro-Kjeldahl technique using 'Kjeltec' equipment (Perstorp Analytical Ltd, Maidenhead, UK). Neutral detergent fibre (NDF) was determined as described by Van Soest *et al.*[23] and acid detergent fibre (ADF) was analysed according to the method of Van Soest and Wine[24] using the Tecator Fibretec System equipment (Tecator Ltd, Thornbury, Bristol, UK). For the PPO activity assay, plant tissue was extracted according to the method of Winters and Minchin[25] and assayed according to the method of Robert *et al.*[26] In brief, leaf material (~0.5 g FW) was extracted at 4 °C in 2 mL of McIlvaine buffer (pH 7) containing 0.1 mol L^{-1} ascorbic acid to inhibit PPO activity. Extracts were centrifuged at 15 000 × *g* for 10 min at 4 °C and the supernatant was retained. Supernatants were desalted by applying to columns (1.5 × 6 cm) containing bio-Gel P6DG (Bio-Rad, Hemel Hempstead, UK) prepared in McIlvaine buffer (pH 7) and centrifuging at 2500 × *g* for 6 min at 4 °C. Active PPO content was determined spectrophotometrically at 420 nm using 10 µL of eluted fraction with 15 µL of 0.001 mmol L^{-1} copper sulfate, 10 mmol L^{-1} methylcatechol and 1.5 mL of McIlvaine buffer. Total PPO activity (active + latent) was calculated with the addition of 0.25% SDS in the McIlvaine buffer. Enzyme reaction rate was defined as the amount of enzyme that produced 1 µmol of quinone per second (µkatal) based on the absorption at λ 420 nm of a known concentration of quinones formed through the reaction of methylcatechol and sodium periodate,[27] giving a conversion factor of µkatal = (0.0453 × Δ optical density).

Calculations and statistical analysis

Lipolysis was calculated as the proportional loss of GBM lipid and not as total net loss of esterified lipid across all classes (DAG + MAG + TAG). This approach is used in 'living' tissue where enzymes such as acylCoA:*sn*-1,2-diacylglycerol acyltransferase upregulate during periods of stress and convert DAG and MAG released during the breakdown of GBM lipid into TAG.[8] Therefore an equation which takes into account the net loss of all esterified lipid will underestimate considerably the lipolytic action of the tissue in degrading GBM lipid. This approach with 'living' tissue has been reported previously[8,10,28] and is calculated according to the following equation:

$$\text{Lipolysis} = (\text{GBM lipid}_{T0} - \text{GBM lipid}_{Tx})/\text{GBM lipid}_{T0}$$

where T = time; 0 = 0 h; *x* = 2, 6, or 24 h.

Lipolysis was analysed using a general analysis of variance with red clover line (PPO+ *vs.* PPO−) × lipid state (PC *vs.* FL) as the treatment. Biohydrogenation was calculated according to the following equation:

$$\text{Biohydrogenation of C18 PUFA} = (\Sigma\text{C18 PUFA}_{T0} - \Sigma\text{C18 PUFA}_{Tx})/\Sigma\text{C18 PUFA}_{T0}$$

where ΣC18 $PUFA_{T0}$ = sum of all C18 PUFA at time 0 h; ΣC18 $PUFA_{Tx}$ = sum of all C18 PUFA at time 2, 6 or 24 h.

Biohydrogenation was analysed statistically using a general analysis of variance with red clover line (PPO+ *vs.* PPO−) × lipid state (PC *vs.* FL) as the treatment. Ammonia-N production and release of FAA were used to predict proteolysis in the PC treatments and were analysed as described for lipolysis. Corrected counts of immunogold label per chloroplast were analysed using a general analysis of variance with cell type (spongy *vs.* palisade) and cell state (fresh *vs.* stressed) as the treatment. All statistical operations were performed with Genstat 8.1 (Lawes Agricultural Trust).[29]

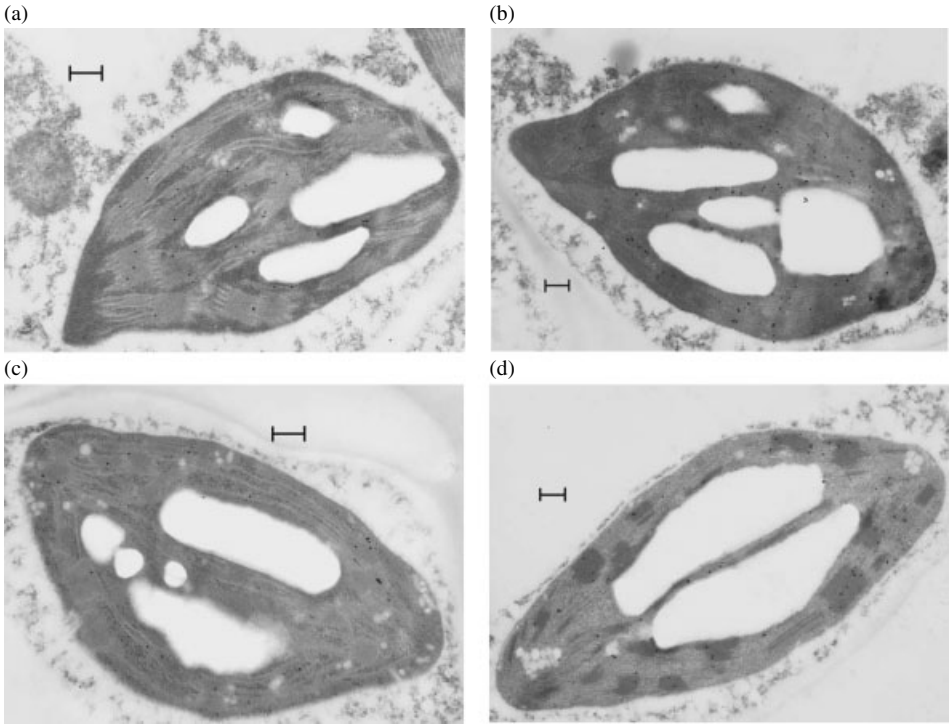

Figure 2. Transmission electron micrographs of (a) fresh spongy mesophyll chloroplast with 29 gold labels, (b) stressed spongy mesophyll chloroplast with 123 gold labels, (c) fresh palisade mesophyll chloroplast with 23 gold labels and (d) stressed palisade mesophyll chloroplast with 66 gold labels. The polyphenol oxidase protein is recognized by a monoclonal antibody conjugated to 15 nm gold particles and appears as black spots. The gold label is localized predominantly on the grana stacks and stroma thylakoids of the chloroplasts. Occasional gold labels are found associated with white starch grains. Bar = 200 nm.

RESULTS AND DISCUSSION

Chemical analyses, polyphenol oxidase activity and localization

Chemical compositions of the wild-type and PPO-silenced red clovers[11] were similar, with 178 and 168 g DM kg^{-1} FW, 915 and 921 g OM kg^{-1} DM, 44.8 and 41.1 g N kg^{-1} DM, 317 and 277.0 g NDF kg^{-1} DM, 150 and 198 g ADF kg^{-1} DM, 74.0 and 80.0 g WSC kg^{-1} DM, 0.863 and 0.860 dry matter digestibility for PPO+ and PPO−, respectively. The PPO activity was significantly different between wild-type and PPO-silenced red clover: 32.5 and 0 μkatal g DM^{-1} for total PPO (latent + active) and 2.25 and 0 μkatal g DM^{-1} for active PPO for PPO+ and PPO−, respectively. The use of transgenic PPO-silenced material,[4,11] as opposed to studies with mutant lines with low PPO activities,[8,10,28] reduces the potential differences in isoenzyme activity and chemical composition between the two lines.

Figure 2 shows a representative electron micrograph of fresh and stressed spongy and palisade mesophyll chloroplast, where immunogold labelling has been used to identify PPO protein. Higher PPO signals were detected ($P < 0.001$) within the chloroplasts of spongy mesophyll cells than palisade mesophyll cells in both freshly cut (40.6 and 25.6 Au label per chloroplast, respectively) and stressed material (cut/crushed and wilted for 1 h; 94.5 and 61.6 Au label per chloroplast, respectively). The higher level of detection ($P < 0.001$) in stressed as opposed to fresh material (×2.37 mean across both cell types) may represent a defence response. Imposed stress such as cell damage has been shown to result in increases in PPO activity, presumably through activation of the chloroplastic enzyme by endogenous, presumably vacuolar diphenol substrates (diphenol).[2,30] In the present study whole chloroplasts were examined and PPO detected and localized by Au labelling. Because the examined organelles were intact, with minimal mixing of enzyme and substrate, the increased level of Au labelling may suggest an increase in the transcription of PPO genes and subsequent translation and import of PPO protein into chloroplasts. Upregulation of PPO gene expression has been reported previously during periods of stress for a number of species[31–33] and it seems logical that such an increase would lead to a greater level of PPO protein translocated to the chloroplasts. However, as the antiserum used in this study was derived from PPO protein that was fully denatured and purified by SDS–polyacrylamide gel electrophoresis,[4] stronger Au labelling could well be due to conversion from latent to active forms, as many, if not all, described mechanisms of PPO activation are thought to involve partial unfolding or refolding of the protein which would expose additional epitopes for antibody binding. While mixing of PPO with its *o*-diphenol substrate has been shown to be capable of carrying out this activation,[2] other mechanisms such as solubilization, inter-conversion,[34] chemical modification such as intermolecular disulfide bridge formation, glycosylation, phenolic glucosides,[35] proteolytic activation[36] and the dissociation of an enzyme–inhibitor complex[37] have all been proposed to account for the activation of latent PPO. The higher level ($P < 0.001$) of PPO protein in the chloroplasts of spongy mesophyll compared with palisade mesophyll (×1.53 mean across fresh and activated cells) may be related to the location of the cell in the leaf: as the spongy mesophyll cells are associated with stomata they are more likely to be subject to pathogenic attack (e.g. fungus, viral or bacteria) and so may require higher levels

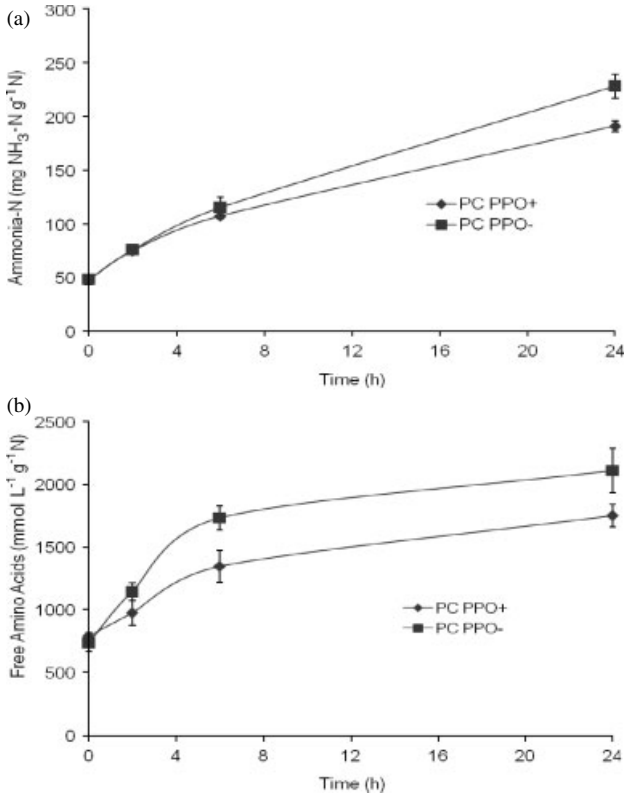

Figure 3. Predictors of proteolysis in the protein complex (PC) treatments with (+) and without (−) PPO activity incubated for 24 h at 39 °C in the presence of rumen fluid: (a) ammonia-N; (b) free amino acids.

of PPO as a defence response than palisade mesophyll, which is located under the upper epidermis layer of cells away from the stomata.

Proteolysis

Figure 3 reports two predictors of proteolysis: liberation of ammonia-N and free amino acids. Both predictors show a higher ($P < 0.05$) proteolytic activity in PPO− than PPO+ after 24 h, showing the effect of PPO in reducing proteolysis in a rumen-like environment. PPO− showed higher levels of free amino acids than PPO+ as early as 2 h ($P < 0.1$) and 6 h ($P < 0.05$), whereas for ammonia-N differences were only apparent after 24 h, which may suggest an initial greater sequestration of amino acids by microorganisms on the PPO− treatment which prevented their degradation to ammonia. Protection of protein by PPO is related to the formation of quinones produced by the catalysed oxidation of diphenols. The exact mechanism of the subsequent quinone–protein reaction is not fully understood. It has been shown that the amine group of free amino acids reacts with quinones, forming phenols that are susceptible to further oxidation reactions.[37] Peptides contain certain side chains, namely amines (e.g. lysine)[37,38] and sulfo (e.g. methionine).[1,37] These have been highlighted as potential targets of quinone attack. Once bound, the quinones reform phenols covalently bound to the protein, and may provide the mechanism by which protein–phenol complexes are formed. The protein–phenol complexes likely reduce proteolysis in silage through direct deactivation of plant proteases[11,39,40] and/or making protein unavailable to proteolytic degradation.[11] Plant enzymes have been shown to have an impact during the early stages of proteolysis in rumen-like conditions.[8,41] However,

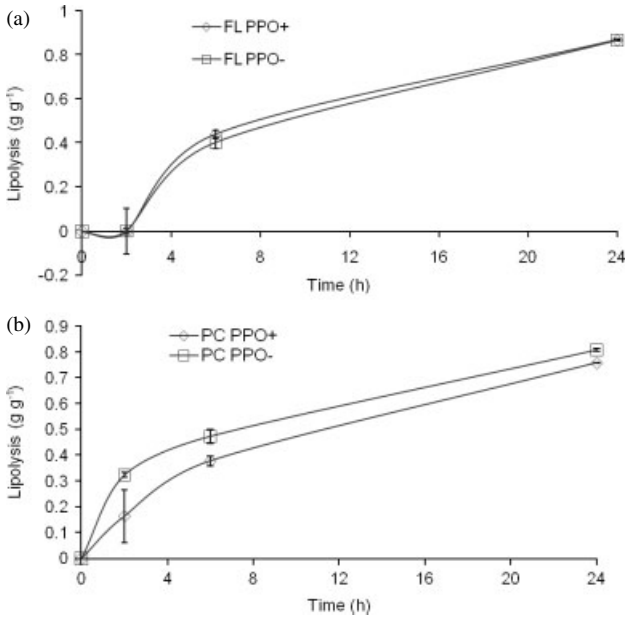

Figure 4. Lipolysis in red clover with (+) and without (−) PPO activity incubated as free lipid (FL, a) or as a protein complex (PC, b) for 24 h at 39 °C in the presence of rumen fluid.

Table 1. Lipolysis and C18 PUFA biohydrogenation (g/g) for red clover with (+) and without (−) PPO activity incubated for 24 h at 39 °C in the presence of protein complexes (PC) or as free lipid (FL)

	PC		FL			*P*		
	+	−	+	−	SED	+ *vs.* −	PC *vs.* FL	Int.
C18:2	0.52	0.58	0.82	0.82	0.014	*	***	**
C18:3	0.81	0.86	0.97	0.97	0.016	*	***	**
Lipolysis	0.76	0.82	0.87	0.87	0.032	*	*	*

SED, standard error of the difference; + *vs.* −, PPO effect; PC *vs.* FL, lipid state effect; Int., interaction effect.
* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

as bacterial colonization of the forage proceeds, proteolysis in the rumen will be increasingly governed by microbial proteases which could be retarded by PPO-induced complexing of leaf proteins, resulting in reduced digestibility.[25] It is entrapment of GBM lipid within these protein–phenol complexes that we hypothesize is responsible for the reduction in lipolysis in the rumen, although an initial role of PPO-reducing plant-mediated lipolysis in fresh forage could also contribute to the effect.

Lipolysis and C18 PUFA biohydrogenation

Lipolysis during 24 h incubations is shown in Fig. 4 and means after 24 h for both lipolysis and C18 PUFA biohydrogenation are given in Table 1. There were no differences in lipolysis between PPO+ and PPO− during the FL incubations (Fig. 3(a)), whereas for the PC treatment PPO+ lipolysis was consistently lower ($P < 0.05$) than PPO−, indicating greater protection of GBM lipid during the course of the incubation. This lower level of lipolysis resulted in a lower biohydrogenation of C18:2 and C18:3 after 24 h, as reported previously for studies utilizing wild-type and low-PPO mutant red clover.[10]

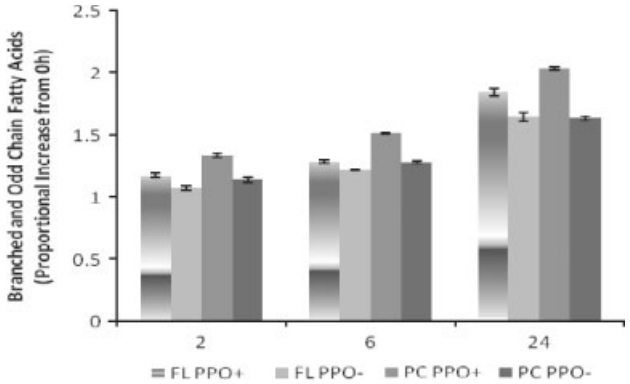

Figure 5. Branched- and odd-chain fatty acid proportional increase from 0 h incubations.

Initial studies on the role of PPO in decreasing the breakdown of GBM lipid (phospholipids and galactolipids) indicated a plant-mediated response[8,42] such as deactivation of plant lipases. Indeed, an inhibitory effect of grape seed extract (containing PPO) on lipases was previously reported.[43] The greater differential between PPO+ and PPO− in the PC treatment of the current study for the earlier time point (2 h) may reflect this reduction in plant-mediated lipolysis. However, during the later time points (post 6 h) microbial lipolysis will become the major catabolic factor and, while the difference between PPO+ and PPO− becomes reduced, there is still evidence for protection from non-plant-derived lipases. Indeed, more recent work has demonstrated that PPO also protects plant GBM lipids from ruminal microbial lipase activity,[10] suggesting either an alternative or additional mechanism to deactivation of plant lipases. This mechanism is unlikely to involve PPO-mediated deactivation of microbial lipases since PPO cannot function in the anaerobic conditions of the rumen. Huws *et al*.[44] indicated that red clover silage feeding as opposed to grass silage feeding resulted in a significant change in the rumen microbial ecosystem within a 2-week period. Such changes could represent adaptation of the rumen microbial population to increase utilization of PPO-protected protein and GBM lipid. Vlaeminck *et al*.[45] reported that branched- and odd-chain fatty acids are potential markers of changes on rumen microbial populations. Using the proportional increase of these fatty acids as an indicator in the current study (Fig. 5) we showed similar shifts as a result of PPO activity, where PPO+ showed proportionally higher levels of these fatty acids ($P < 0.001$) than PPO− for both FL and PC incubations. This effect was seen to a greater extent in the PC treatment compared to FL ($P < 0.001$), which suggests that PPO-induced changes in nutrients other than just GBM lipid result in greater changes in the microbial community. However, recent evidence[46] has indicated that shifts which occur in the microbial population as a consequence of red clover feeding did not result in a greater utilization of PPO-protected protein or GBM lipid. In addition, the active constituent of red clover in reducing C18 PUFA biohydrogenation was PPO and not a consequence of the microbial population shift as with other dietary regimes (e.g. fish oil).[47] This implies a direct protection mechanism of the GBM lipid molecule, such as interaction with protein–phenol matrices as opposed to alteration of the microbial rumen community, although the importance of reduced plant-mediated lipolysis cannot be ignored.

This study provides the first evidence that PPO protection of GBM lipid in the rumen may be related to lipid entrapment within protein–phenol complexes as the separation of lipid from PPO-activated forage substrate resulted in loss of protection of the GBM lipid from lipolytic activity in rumen fluid cultures. However, more detailed investigation is required to categorically assign the mechanism. Although no evidence has yet been described for direct GBM lipid–phenol binding, there are no obvious nucleophilic sites on GBM for PPO-generated quinone attachment. However, removal of the lipid from red clover tissues through the use of organic solvents (as in the experiment here) could hypothetically result in changes to a phenol-bound lipid structure and as such lose protection. Therefore isotope marker studies will be required to definitively determine whether phenol-produced quinones bind with GBM lipid and to determine whether lipids interact with protein–phenol complexes.

CONCLUSIONS

PPO protein was detected to a greater extent in spongy as opposed to palisade mesophyll cells of red clover leaves. Damage to the leaves resulted in over a doubling in PPO labelling found within mesophyll cells, potentially as a consequence of conversion from latent to active PPO without complete organelle rupture. Reduction in microbial lipolysis of GBM lipid through the action of PPO was apparent in macerated red clover tissue but no protection was observed if the lipid was incubated in the absence of the cellular matrix. This observation is consistent with a mechanism for the ruminal protection of GBM lipid by PPO through entrapment of lipid within protein–phenol complexes rather than by covalent modification of lipids by PPO-generated quinines.

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